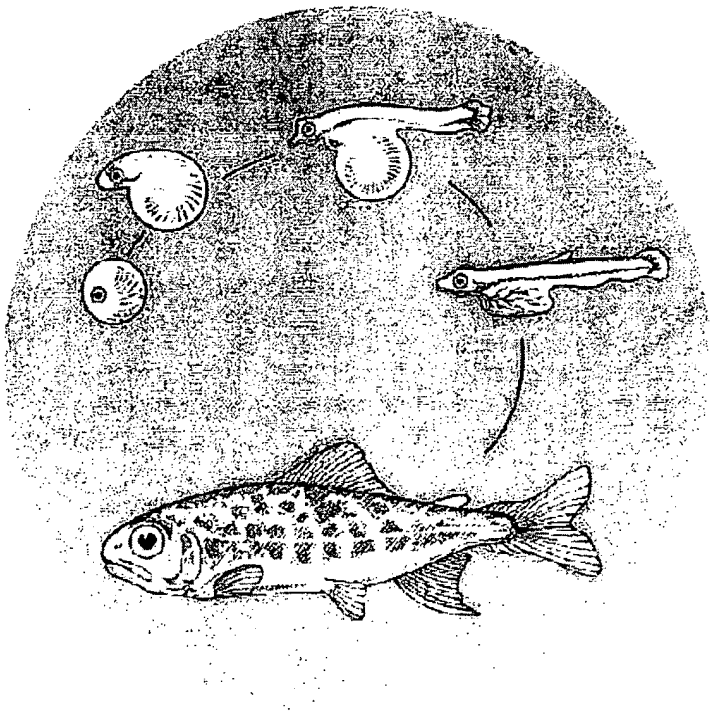
Exhibit J

October 1986

ETIOLOGY OF EARLY LIFESTAGE DISEASES

Final Report 1985



DOE/BP-18186-1

This report was funded by the Bonneville Power Administration (BPA), U.S. Department of Energy, as part of BPA's program to protect, mitigate, and enhance fish and wildlife affected by the development and operation of hydroelectric facilities on the Columbia River and its tributaries. The views of this report are the author's and do not necessarily represent the views of BPA.

This document should be cited as follows:

R. W. Sauter, C. Williams, B. Celnik, E. A. Meyer - Dept.of Microbiology and Immunology, Oregon Health Sciences University, Etiology Of Early Lifestage Diseases, Final Report 1985, Report to Bonneville Power Administration, Contract No. 1984BI18186, Project 198404400, 53 electronic pages (BPA Report DOE/BP-18186-1)

This report and other BPA Fish and Wildlife Publications are available on the Internet at:

http://www.efw.bpa.gov/cgi-bin/efw/FW/publications.cgi

For other information on electronic documents or other printed media, contact or write to:

Bonneville Power Administration
Environment, Fish and Wildlife Division
P.O. Box 3621
905 N.E. 11th Avenue
Portland, OR 97208-3621

Please include title, author, and DOE/BP number in the request.

ETIOLOGY OF EARLY LIFESTAGE DISEASES

Project 84-44

Final Report

R. W. Sauter
Instructor

C. Williams
Sr. Research Asst.

Dept. of Cell Biology
and Anatomy

E. A. Meyer
Professor

B. Celnik
Sr. Research Asst.

Dept. of Microbiology
and Immunology

Oregon Health Sciences University
Portland, Oregon

In Collaboration With
Abernathy Salmon Culture Technology Center
Longview, Washington

ABSTRACT

Each year hatcheries experience loss of eggs, fry and fingerlings due to a group of poorly defined diseases called White Spot and Coagulated Yolk. Studies by other investigators generally attribute these diseases to water chemistry, rough handling or water flow rates. Only a few brief reports are found suggesting bacteria as a potential cause of these diseases.

To test the hypothesis that bacteria are etiologic agents causing early life stage diseases, groups of eggs were incubated and reared in separate lots with mortalities recorded. Samples of maternal blood and ovarian fluid (coelomic fluid), as well as unfertilized eggs, were collected at spawning and subsequently tested for the presence of bacteria. Our tests reveal that there is a wide range in mortality rates experienced by the progeny of different brood salmon. During two brood years, it was found that 50% to 75% of the mortality total for the 30 egg lots tested each year were produced by only 6 of these egg lots. Microbiologic tests revealed that these eggs contained a variety of both Gram positive and Gram negative bacteria within their yolk and that the fluids from the females who produced these lots were contaminated with a variety of bacteria. In contrast the eggs and maternal fluids from the six egg lots which experienced the lowest mortalities did not contain high numbers of Gram positive bacteria and contributed only 5% of the total mortalities observed within the 30 egg lots tested in both years.

From the 60 egg lots tested over two brood years we have isolated 18 different bacterial genera containing 32 different species from within the yolk of surface sterilized. unfertilized eggs. Our tests strongly suggest that Aeromonas hydrophila, Pseudomonas (3 species) Staphylococcus aureus, Vibrio sp., Corynebacterium hoffmanii, Listeria sp. and Bacillus SP. when detected within the yolk of eggs sampled from egg lots prior to fertilization will be associated with higher than normal mortality rates when the remainder of the egg lots containing these bacteria are incubated and reared. Four of these bacterial genera are gram positive and three are gram negative. Multifactorial disease processes involving

relationships between hemolysin producing bacteria and endotoxin producing bacteria are suspected.

INTRODUCTION

Each year in the Columbia River system, several hundred million salmon eggs are spawned and the fry reared in hatchery facilities. In certain cases many of these eggs or the resulting fry die during their early lifestages from poorly defined diseases which are generally termed "Whitespot", "Coagulated Yolk", Pinhead", "Fin Rot", and "Soft Shell". The severity of losses due to these diseases varies from year to year and from hatchery to hatchery. Wolf (1970) states that Whitespot disease is a symptom of abnormality within fish eggs and fry and supports the belief that chemical factors are the most important causes of this disease. He states that any bacteria within the yolk of eggs or fry are most likely to be secondary to the primary cause which is chemical.

Leach (1924) contended that rough handling of incubating eggs caused Whitespot while Agersborg (1933) believed that low oxygen levels would induce the disease. Treatment of incubating eggs with drugs or other chemicals was proposed (Mazuranich and Nielson, 1959, Wood, 1974) as inducers of the disease. MacKinnon (1969) proposed mineral deficiency in the incubation water as a causal factor. Wood (1974) believed that supersaturation of incubation water with gases was the cause of Whitespot and Coagulated Yolk. Of these suggested causes, none could consistently induce Whitespot or Coagulated Yolk. Banks (1983) demonstrated that the use of artificial substrate in vertical incubators could reduce the incidence of "Coagulated Yolk" in fall Chinook Salmon (Oncorhynchus fshawytscha) but would not eradicate the problem. He also observed that offspring from some salmon were more susceptible to Coagulated Yolk than offspring from other salmon.

The presence of bacteria within the yolk of incubating eggs and sac fry has been reported sporadically. Von Betegh (1912) reported Diplo-bacillus liauefaciens Pisum to be present in fry suffering from "blue sac disease." Gulberlet et al. (1931) isolated a facultative anaerobe which was Gram negative and motile which they felt was similar to the bacterium isolated by Von Betegh (1912). Cone (1982) reported the presence of a Gram positive bacterium (LactobacilluS sp.) in eggs of Salmo pairdneri. Of great significance arc the

reports of Bullock et al. (1978) and Evelyn et al. (1984) of the presence of the causative organism of bacterial kidney disease (Renibacterium salmoninarum) within eggs taken from female salmon (Oncorhynchus kisutch) which were known to have this bacterium present within their coelomic (ovarian) fluid. These reports demonstrate the vertical transmission of bacterial disease from adult diseased salmon to their progeny via the yolk of the eggs which the female produced.

Prior to initiation of this project, three years of preliminary studies allowed us to develop and test a protocol for monitoring groups of progeny of individual salmon during the hatchery rearing process. This protocol identified several important areas of concern in the etiology of early lifestage diseases which involved bacteria within the yolk of eggs as the primary cause of these diseases. Our preliminary studies revealed that:

A. The mortality experienced by the progeny of different female salmon varied greatly. Some salmon produced eggs which experienced very low overall mortality (less than 5% from fertilization through the first 12 weeks on feed) while other females produced eggs which experienced very high mortality rates (70% to 100% during the same interval).

B. The ovarian fluid of some female salmon contained high levels of unknown bacteria of several types. Other females had ovarian fluid which contained few if any bacteria. This suggested that egg and fry mortalities could be due to the vertical transmission of disease from a diseased adult salmon to her progeny via the yolk of her eggs. The vertical disease transmission hypothesis was further supported by the visualization of bacteria within the yolk of many eggs by microscopic examination of histologic preparations of eggs collected at spawning.

C. Preliminary tests revealed that in-hatchery losses could be reduced by water hardening the eggs in a water soluble antibiotic.

D. Losses during the study period ranged from 1/7th to 1/4th of the egg take

depending on the year. Furthermore, there were indications that many of the survivors were disease carriers which could both spread the disease to other healthy fish in the rearing ponds and later succumb to the diseases themselves after release into the wild.

Our preliminary studies revealed that the poorly understood early lifestage diseases are insidious in character and that the disease processes are active and detectable microscopically within the fish long before the external physical symptoms are displayed. These disease processes included pathologic changes in fins, kidneys, and digestive tract within fish which appeared to be healthy as judged by their behavior and general external appearance. These pathologic changes suggested damage from endotoxins produced by Gram negative bacteria.

Based on our preliminary information, this project was designed to isolate and identify the variety of bacteria found in adult blood, ovarian fluid, and yolk of unfertilized eggs. Certain of these bacterial isolates were used to challenge groups of fish to determine which bacteria are pathogenic and to identify the organs which they damage. The bacterial isolates were also tested for drug sensitivities to identify a group of antibacterial agents for potential use in the water hardening process to eradicate the bacteria located within the yolk of salmon eggs. Since endotoxins exert detrimental effects on mammalian physiologic and immune functions, this bacterial product was tested for in ovarian fluid and egg yolk samples.

MATERIALS AND METHODS

The objectives of this project were: (1) to assess the concept of vertical disease transmission, (2) to identify bacteria isolated from adult female salmon blood and ovarian fluid, and from within the yolk of the eggs she produced, (3) to determine the variation in mortalities experienced by groups of eggs produced by different female salmon, (4) to establish which of the many bacterial species are associated with higher than normal mortality rates, and (5) to determine the levels of endotoxin in ovarian fluid and yolk of

unfertilized eggs. Furthermore, histologic preparations of adult, embryonic and juvenile salmon were prepared to detect the earliest onset of pathologic processes. Bacterial challenge tests were conducted to identify which of the bacterial isolates produced morbidity or mortality, and the challenged specimens have been subjected to histologic examination to determine which body organ(s) are most severely damaged by the various bacteria.

Mortality Study

In brood years 1984 and 1985, the first 30 adult fall chinook salmon (Oncorhynchus tshawytschaj judged to be ripe for spawning on one day were used in the mortality study. This group of 30 fish comprised 3/4th of all the fish which were ready for spawning on that day. The females were spawned and their eggs fertilized from a reservoir of sperm pooled from 8 males. Thus, variations in subsequent mortality rates within the 30 groups of eggs in all probability resided within some parameter of the female component of the test. The eggs from each female were placed into individual identifiable trays which had been soaked in Wescodyne for one hour prior to use. The eggs were incubated in constant temperature (12° C) well water. Each tray of eggs was placed in the top row of stack incubators so that the single pass water would not come into contact with other incubating eggs prior to reaching the group of the test eggs. Records of mortalities were maintained from fertilization through the first 12 weeks on feed. When the fish were ready to be fed. each group of fry was subsampled. The 300 fish from each female were maintained in separate aquaria to determine the mortality for each group during the first 12 weeks on feed. The Abernathy Dry Diet (Fowler, 1980, 1981) was used for feeding.

During incubation and rearing, samples of unfertilized, green, and eyed eggs were

---

*All references to "fish" or "Salmon" hereafter refer to only this genus and species unless otherwise noted.

taken. Half of the sample from each group of eggs was frozen for future microbiologic testing; the other portion was preserved in either Alcohol Formol Acetic Acid fixative or Phosphate buffered formalin for histologic examination. At the completion of this phase of the project, all remaining fish were placed into the production rearing facilities for ultimate release as part of the general hatchery production.

Establishment of Mixed Bacterial Cultures

At spawning, samples of adult blood, ovarian fluid, and unfertilized eggs were collected aseptically from each of the thirty female salmon, inoculated into Fluid Thioglgcollate medium and the resultant organisms subcultured, isolated and identified. This is described below and summarized in figure 1.

Blood samples were collected by passing a syringe needle through the body wall, which had been previously surface sterilized, and into the ventricle of the heart. After blood flow was established, a presterilized, EDTA coated vacutniner was attached to the needle, and the vial was filled with blood. A 0.5 ml sample of this blood was inoculated into blood serum culture bottles containing thioglycollate medium. The inoculated serum bottles were incubated at 6° C for a week before samples were streaked onto plated media (MacConkey Agar, Blood Agar, Brucella Agar, and Chocolate Agar) which began the isolztion and identification of the various bacteria using standard microbiologic techniques (Sec Fig. 1). All media used in these experiments were obtained from Prepared Media Laboratories, Tualatin, Oregon.

Ovarian fluid samples were collected in a manner similar to that used in collecting blood. However. these vacutainers were not coated with EDTA. Promptly after collection. 0.5ml aliquots of ovarian fluid were inoculated into blood serum culture bottles containing thioglycollate, and vials of Leptospira Broth. The inoculated media were maintained at 6° C for one week, then examined for microbial growth. Samples of the fluid media were then streaked onto plated media 3s above (see Fig. 1) to commence the isolation and identification of the various bacterial types. The remaining ovarian fluid was stored for

future study at -70' C.

To culture bacteria from the yolk of unfertilized eggs, the following protocol was used. The egg surfac was sterilized by being dropped into 3 mixture of 0.05% aqueous iodine and allowed to react for 3 minutes. The eggs were rinsed in three changes of sterile water to remove residual iodine. Four surface sterilized eggs per vi31 were placed intact into fluid thioglycollate 3nd incubated at 6° C for two weeks. At the end of this time the fluid surrounding the intact eggs was Gram stained and examined microscopically for the presence or absence of bacteria. The sterility of the media was further tested by streaking samples of the medi3 onto Tryptic Soy Agar plates. After confirmation of sterility by testing for the absencce of bacteria ouside the chorion of the eggs, the eggs were aseptically crushed and the yolk mixed into the media. Following one week of incubation, samples of any resulting microbial growth were streaked onto plated media. Any growth of bacteria subsequent to crushing the eggs was of bacteria known to have been within the egg at spawning time.

Addition31 tests of the yolk and ovarian fluid samples by direct plating onto Cytophaga media and TSA with 5% sheep blood were conducted to isolate any organisms which might not survive the thioglycollate enrichment procedure. All isolates were identified by initial determination of Gram reaction, morphology, 3nd subsequent determination of appropriate biochemicla characteristiecs. Our reference for idenitifying bacterial gener3 nnd species is Bergeys Manual of Determinative Bacteriology (1974). These isolations were conducted under aerbic, anaerobic 3nd elevated carbon dioxide conditions. All isolstcs have been preserved on cultue medium slants for further study. Selcctcd culturcs have been sent to reference laboratories for confirmation of identifications.

Tests for fungi have been done using Sabouraud's a n Mycosel medi3. No fungal isolated have been obtained rrom any speciments to date.

Endotosin Assay

Using the Limulus amebocytc lysatc test (Levin et al., 1968, 1970), the levels of endotoxins in samples of ovarian fluid nnd yolk of unfertilized eggs collected at spawning were determined. The samples collected during the 194 brocd year were tested by the gel forming procedure (Associates of Cape Cod) in which the gel firmness is proportional to the concentration of endotoxin within the sample. The samples collected in the 1985 brood year were tested with the chromogenic assay (Whittaker M. A. Bioproducts) which gives a colored product which can be read on an ELISA reader, the absorbance being proportionsl to the concentration of endotoxin within the sample.

Bacterial Challenges

In both years of this project bacterial challenges were conducted in efforts to identify which of the several species of bacteria were the most detrimental to the developmemt of the parr stage salmon used in the test. The bacteria uded in these challenges were isolated from the yolk of surface sterilized, unfertilized eggs which had been sampled from egg lots which experienced higher than normal mortalities during incubation and early rearing. The challenge organisms were suspended in physiclogic saline and injected subcutaneously near the base of the dorsal fin.

The challenges during the first year (1984) were designed to be short term (2 weeks) acute tests in which high doses of bacteria were given at dilution 1 and other groups of fish received 5 fold serial dilutions of the inoculum. The purpose of this challenge series was to identify, in a short period of time. which of the bacterial species tested caused nortalities and/or pathologic changes in liver. kidney, spleen or digestive system. and to identify which of the test organisms merited further testing in long term chronic challenges. The bacterial species identified for further testing were Aernmonns hydeophila. Pseudomonas fluorescens. Pasteurelln multocidn. Vibrio parnhemolviius and Listeria sp. (a beta hemolytic strain).

In 1985, the above bacterial species were used in chronic tests at dose levels of 10^3 and 10^6 viable bacteria per fish. Groups of 50 fish were given the inoculum at the base of

the dorsal fin. placed in aquaria and monitored for mortalities over an 8 week period. At three weeks post-injection the test fish were anesthetized. weighed and measured. Specimens were collected for pathologic examination and blood samples taken to use in attempts to recover the challenge organism. To prevent overcrowding during the remainder of the test, only 25 of the challenged fish in each group were returned to their aquaria. At the end of the challenge period the remaining fish were again anesthetized, weighed and measured. Samples of blood were taken to test for the presence of the challenge organism and samples were preserved for pathology studies. The test fish used in the chronic challenges were the progeny of the two female salmon whose eggs had experienced the lowest mortalities during incubation and rearing (egg lots 1 and 3).

During the '84 brood year, one egg lot in the mortality study experienced heavy soft shelling. Since our preliminary studies several years prior to initiation of this project had revealed that soft shelled eggs had chorions with bacterially laden areas of erosions and pitting, these soft shelling eggs were incubated in thioglycollate medium and from the resulting growth bacteria were isolated and identified. Similar samples were obtained from Spring Creek NFH and the bacteria on the surface of these eggs were isolated and identified. Acromonas hydrochila. Entrobacter agglomerans and Klebsiella Dneumonae were species common to both Abernathy SCTC and Spring Creek NFH samples as well as the genus Pseudomonas which was represented by four species, none of which were common to the samples from the two hatcheries.

To test if the isolates described above could induce soft shelling the eggs of three female salmon were fertilized from a common pool of sperm. rinsed briefly and placed into mini incubators containing well water to which hnd been added known concentrations of the three organisms common to Abernathy and Spring Creek soft shell specimens and Pseudomonas fluoresccns. The challenge dose levels were at 10^3 and 10^6 bacteria/cc of well water in the incubator, the eggs were allowed to water harden in the incubators for 30 minutes to allow time for the bacteria to attach to the surface of the chorion if possible.

After 30 minutes the bacteria were flushed from the incubators into a chlorine treatment system and the eggs were allowed to incubate normally. Samples for histologic examination and microbiologic testing for the challenge organisms were collected at eye up and just prior to hatch.

RESULTS

Mortality Study

The cumulative mortalities experienced by the progeny of individual brood salmon which were followed from fertilization through the first 12 weeks on feed are shown in figure 2 A,B. The average of the mortalities experienced by the 1984 progeny groups was 16.98% (range 2.85%~ to 100%) whereas the 1985 brood year progeny groups experienced an average mortality of 34.760/6 (range 6.93% to 96.18%). In brood year '84 it was seen that 73.35% of all the mortalities during incubation and rearing were from the progeny of 6 of the 30 females tested. In contrast, 5.4% of the mortalities came from the 6 lowest mortaaliy progeny groups. In brood year '85 the 6 highest mortality progeny groups produced 38.87% of the mortalities experienced by all the 30 groups tested, whereas, the six lowest mortality groups produced only 5.95% of all the mortalities experienced by the 30 test groups This suggests that the eggs produced by 3s few as20% of the female salmon spawned in 3 given year can account for 40-75% of all the mortalities experienced during incubation and early rearing.

During incubation it is seen that certain progeny groups experience thir mortaliteds at different times. Some progeny groups experience higher than average mortalities during the green egg stage (fig. 3. .A,B) then experience very low mortality rates for the remainder of incubation and early rearing. Other progency groups experience very low green egg mortality followed by high mortality rates during the eyed egg stag:: (fig. 4. -4-D). For comparison, the mortality graphs of the two lowest mortality progeny groups arc shown in Figure 5. When moribund eggs were picked from the various progeny groups and the yolk

of these eggs was plated onto TSA with 5% Sheep blood. plate counts of 10,000-100,000 or more viable bateria per cc of yolk tested were made in 27 of the 30 egglots. Histologic examination of' moribund eggs further confirmed the common presence of bacteria within the yolk of these eggs. The moribund eggs used were seicctcd on the basis that they were omly slightly. off color and had no grossly visible fungusing. The high incidence of bacteria within moribund eggs as demonstrated by bacterial culture methods and histologic examination support the belief thal mortalites during incubation and early rearing involve bateria as etiologic factors in the observed mortalities.

Results: Bacterial isolations

From sixty samples each of adult blood. ovarian fluid 3nd the yolk of surface sterilized unfertilized eggs collected over 2 brood years, 21 genera of bacteria were isolated and 46 species were found within these gcncr3 (Table 1). Based on frequency of detection, 62 of the 180 samples contained Aeromouas hvdrnphila, 35 contained Listeria sp., 16 contained pscudomnnas fluorescens and 15 contained a highly drug sensitive strain of Staphylococcus aureus In additoin. 37 samples contained Pseudomonas species other than fluorescens 3nd 16 samples contained varios species of enterobacter. Cornebacteruim hoffmaini was detected in 7 samples and Bacillus sp. was detected in 5 samples. Other species were detcctcd occasionally. Tests for Myxobacteria and fungus within the damoles were negative.

From the 60 egglots tested for bacteria within the yolk of surface sterilized unfertilizcd egg:. 42 of these egg lots contained detectable bacteria. These isolatiuns were obtained following enrichment of the samoles in thioglycollate. Table 2 lists, in order of frequency of isolation. the genera of bacteria isolated from the yolk of unfertilized eggs over a two year period. The first seven gener3 listed comprise 75% of 311 the isolations made from the egg lots tested. Eleven other gcner3 somprisc the remaining 25% of the isolations. Interestingly, four of the seven most commonly detected bacterial species are gram positive organisms.

The efficiency of thioglycollate enrichment for the detection of bacteria which are present either in low numbers or are fastidious in their growth requirements is shown in figure 6. In this test only 6 of the twelve egglots shown yielded bacterial growth by direct plating of the samples onto culture media without prior enrichment. The other 6 samples tested as being apparently sterile. However, when these samples were enriched in thioglycollate broth, all tested positive for bacteria. Samples of yolk from all progeny groups in the 1985 brood year were tested for bacterial growth by direct plating and plating following enrichment. It was found that 16 of the samples grew bacteria by direct plating and 14 did not. Plating following enrichment yielded bacterial growth from all 30 egg lots. With the exception of Staphylococcus aureus and one isolation of Listeria sp. the bacteria isolated by direct plating were a great variety of gram negative organisms.

Because so many genera and species of bacteria occured within the various egg lots, it was found that more meaningful information concerning potential associations of bacteria with mortality rates could be obtained by comparing the bacterial types isolated from within eggs sampled from lots which were at the extreme ends of the mortality graphs (Fig. 2) for each brood year. The bacteria isolated from surface sterilized, unfertilized eggs from the 12 high mortality egg lots (6 lots each year) are shown in figure 7. The 12 lowest mortality egg lots similarly tested are shown in figure 8. The number of egg lots in which various genera of bacteria were detected within these mortality extreme groups are summarized in figure 9.

A relationship of type of hemolysis characteristic of some of the many bacteria isolated from within surface sterilized eggs with subsequent mortality experienced during incubation is shown in figure 10. When the bacterial isolates from certain egg lots were neither alpha nor beta hemolytic the mortality in these egg lots was lower (24.10%) than the average for all egg lots (34.76%). The egg lots with alpha hemolytic isolates experienced mortalities averaging 33.76% and the egg lots with beta hemolytic isolates had mortalities averaging 41.01%. The egg lots with a mixture of both alpha and beta hemolytic isolates had an average mortality more than double (71.06%) the average mortalities for the 30 egg lots tested. Strains of Listeria sp. were the most common isolates with this type of hemolysis. These findings suggest that not all organisms present within eggs have an adverse effect but when either beta, or alpha and beta, hemolytic bacteria in combination arc present, the mortality rate will be much higher.

Our experience leads us to propose the following composite sample for microbiologic testing which would allow for the capture of the greatest variety of microorganisms (fig. 11). In this protocol. adult blood drawn from the ventricle is mixed in a vial containing "clean caught" eggs and ovarian fluid collected as the fish is spawned. The contents of the vial are then thoroughly mixed. From this mixture samples can be directly plated on diagnostic media 3s now practiced in fish disease laboratories; however, another portion can be enriched in thioglycollate follow-cd by plating onto various media to detect organisms which could be missed by direct plating without enrichment.

In brood year 1984, egg lot =1 1experienced 3 high incidence of soft shelling. Samoles of this egg lot and other soft shell samples collected from Spring Creek NFH wcrc incubated in thioglycollate in efforts to culture bacteria from the surface of these diseased eggs.Aeromonas hydeophila Enterobacter agglomerans and Klebseilla pneumounae were isolated commonly found on the surcace of these eggs samoled from two hatcheries. The complctc list of isolated is shown in table 3.

Results: Endotoxin determinations

We have found that the chromogenic assay for endotoxins used in brood year 1985 is superior to the clot forming assay used in 1984 in that the former method is more sensitive and reading of the final reaction product is not subject to judgemental interpretations as is the clot forming assay. All egg lots were assayed for endotoxin levels in the yolk of unfertilized eggs collected at spawning; however, egg lots #7 and #lO were "bad eggs" which would not be used in normal hatchery operations and are not reported. Egg lot #I6 had a very high endotoxin level compared with the other 27 egg lots and has been excluded.

The average of the endotoxin levels in the yolk samples from 27 egg lots was found to be 0.290 [EU (Endotoxin Unit] = 0.1 ng endotoxin). The range was from 0.0 EU to 0.8 I EU among these egg lots. Figure 12 shows the endotoxin levels in egg lots grouped according to bacterial plate counts of yolk sampled from each lot and plated directly onto tryptic soy agar with 5% sheep blood without prior enrichment of the samples. In comparing the group of eleven egg lots which did not yield bacterial growth from the yolk with the group of 13 egg lots which yielded bacterial counts of 10,000 or more it is seen in the latter group that the endotoxin level is 2.6 times that of the group which did not grow bacteria (0.399 EU vs 0.152 EU) and that the green egg mortality is twice as high (10.73% vs 5.22%). When the egg lots are grouped according to percent mortalities experienced during the green egg stage (figure 13) it is seen that as mortality levels increase the endotoxin levels within the yolk similarly increase.

Since there is a general upward trend in endotoxin levels concomitant with increased mortalities during the green egg stage. a comparison of gram negative bacteria isolated from the yolk of surface sterilized, unfertilized egss from the five highest and five lowest green egg mortality groups was made. Enterobacter aglomerans, Hafnia alvei and Moraxella sp. were isolated from only the low mortality egg lots. Pseudomonas sp. was isolated from only the high mortality egg lots. Aeromonas hydephila, Vibrio sp. and Serrntin sp. were isolated from both the high and low mortality egg lots. The single bacterium iso-

lated with high frequency in high mortality egg lots and with low frequency in low mortality egg lots was Listcria sp. 3 Gram positive organism (Figs. 12,13). Grouping of egg lots according to the presence or absence of Listeria sp. (Fig. 13) shows that the egg lots containing this organism have nearly twice the endotoxin level and experience more than twice the green egg mortality than do the egglots which lack Listeria sp. This suggests that this gram positive bacterium may act in synergy with a variety of gram negative organisms to induce a higher than normal green egg mortality. If this is correct, then mortalities during early life stages would be due to multifactorial associations of a variety of both gram positive and gram negative bacteria.

Endotoxin levels in ovarian fluid samples were determined and a range of 0.11 to 15,600 EU/cc of fluid was found. Six of the thirty samples had endotoxin levels in excess of 1,000 EU/cc of fluid. It appears that there is no passive transport of endotoxin from the ovarian fluid across the chorion and into the egg yolk since three of the egg lots had higher endotoxin levels in the yolk than did the surrounding ovarian fluid, also, three ovarian fluid samples with very high endotoxin levels (1510, 1660. 7500 EU/egg) contained eggs with every low levels of endotoxin (0.0. 0.075. 0.088 EU:egg) in the yolk. No correlation of endotoxin levels in ovarian fluid with mortality rates during any stage of incubation and rearing could be acertained. These findings suggest that cndotoxin levels in the yolk of freshly spawned eggs arc due to the presence of bacteria within the yolk, an uptake of exogcnous endotoxin during vitellogcncsis or 3 combination of both.

Two samples of freshly produced fish diets were also tested for endotoxins. The finished dry diet sample was found to contain 6182 EU'g whereas the moist pellet sample contained 4.5 times this amount (27.600 EU/g). Testing of the individual components of the diets revealed that wheat products (33.000-105.000 EC-'g), blood meal (35,000 EU/g) and whey (441,000 EU/g) were the major sources of endotosins incorporated into the diets. Many viable gram negative bacteria were cultured from the finished diets which could be an additional source of endotoxins in the two diets tested.

Results: Bacterial Challenges

The results of the bacterial challenges are shown in Figure 15 in which the challenge organisms are listed in order of decreasing incidence of mortalities. Shown are the average weights and lengths of the survivors, the last two columns present the weight and length as percents of the control group (control = 100%). Aeromonas hyrophola challenged fish had the highest mortality and the survivors showed the least increase in weight (83.3% that of controls) and length (94.6% that of controls). Serratia marcescens and Pasteurella multocida challenged fish also showed a lag in weight and length gain. Fish challenged with Listeria sp. and Pseudomonas fluorescens showed increased weight gain over that of the control groups.

Comparison of the length and weight gains of the fish challenged at 10^3 bacteria/fish with the fish challenged at 10^6 showed, with the exception of Listeria sp., that the lower dose level fish increased more in weight and length than did the fish challenged at the higher dose level, thus a dose response was seen. In the case of Listeria sp., the 10^3 challenged fish, at termination, weighed 89% that of the 10^6 group and a similar lag in growth in length was seen The weight and length data collected at three weeks post injection show that the lag in weight and length increase was more severe at the 10^6 dose level but at termination this trend had reversed and the lag was overcome. In the 10^3 group 3 slight lag in weight and length increase was seen at 3 weeks post injection but it continued to become more severe during the remainder of the test.

Because kidney damage had been seen in tissue samples from the acute challenges conducted the previous year. 311 fish sacrificed at termination of the 1985 chronic challenges were desicatcd to determine what percent of body weight was water. Scattergrams showed in all challenge groups that the higher challenge dose level fish had a higher percent body weight 3s water than did the counterpart low dose groups. The diffcrenccs were not large (0.5-1.7%) but the trend was consistent between dose levels. Thus a dose response was seen, although small.

The bacterial challenges conducted to attempt to induce soft shelling were equivocable. Samples from most of the challenge groups showed some areas of erosion on the surface of the chorion and these areas were seen to contain bacteria. Histologic examination revealed that in no instance were the erosions as extensive as those seen in samples of naturally occuring soft shell. The control groups also showed areas of erosion of the chorion. Bacterial testing of the surfaces of the eggs at eye up yielded the challenge organisms only sporadically among the three replicates of each challenge dose. Furthermore, a variety of waterborne organisms were grown from the egg surfaces.

The fish used in the challenges were from the two lowest mortality egg lots. One of these egg lots contained a non hemolytic strain of Listeria in the yolk of eggs sampled at spawning. Blood samples from late parr stage specimens from this group yielded a non hemolytic strain of Listeria. Similarly, the survivors of egg lot #15, which contained a strongly beta hemolytic strain of Listeria in unfertilized eggs, were found to also contain 3 similar strain of this bacterium in blood sampled in the late parr stage. This indicates that some of the survivors of diseased egg lots are likely to be carrying the bacterium at the time of release from the hatchery.

DISCUSSION

This study demonstrates that there is a wide range in the mortality rates experienced by the progeny groups of individual salmon during incubation and rearing. Bacteriologic testing reveals that there is a great variety of bacterial species present within the yolk of eggs collected at spawning and that some of these species are more commonly found in egg lots which subsequently expericncc uncommonly high mortalities. It appears there arc combinations of bacterial species such 3s Listeria sp.. and Aeromonas Hydeophila (Fig. 16) which are associated with the higher levels of mortality (,56.1%), when these two organisms occur singly within an egg lot the mortality rate is much lower (Approx. 33%). This suggests that much of the early incubation and rearing mortalities are multifactorial in nature

In support of multifactorial disease processes is the finding that egg lots containing the Gram positive Listeri3 sp. also have endotoxin levels produced by Gram negative bacteria which are twice 3s high as that found in non-Listcri3 egg lots. Sullivan et al. (1983) and Jay et al. (1979) have shown that increased endotoxin levels correlate with increased numbers of Gram negative bacteria and therefore. endotoxin levels are measures of Gram negative sepsis.

The detection of Listeri3 sp. in the blood of smolts from egg lots which contained this organism in the yolk of unfertilized eggs indicates that vertical transmission of bacteria does occur and that some of the diseased eggs die while others may survive as carriers. This finding supports the work of Evelyn et al. (1984) and Bullock et 31. (1978) who demonstrated the vertical transmission of Renibacterium salmoninarum. Our detection of Aeromonas salmonicida in two egg lots and Yersinia ruckeri in two other egg lots suggest that these species can also be vertically transmitted, Abernathy SCTC does not have a history of problems with these organisms. It would be worthwhile to test for the frequency of these pathogens in eggs collected at hatcheries with a history of substantial problems aria-ing from these organisms.

Using our enrichment protocols, 18 genera of bacteria have been isolated from within the yolk of eggs collected at spawning. It is seen that high mortality egg lots have 3 frequency of isolation of Gram positive organisms more than three times that of the low mortality groups. In the Gram negntive caterory, the combined isolations of Pseudomonas, Aeromonas, Serratia and Vibrio is twice 3s high in the high mortality egg lots compared with the low mortaliy egg lots. The isolation of Enterobacter, Moraxella, Hafnia and Yersinia do not apperar to be related to early rearing mortalities at this time.

In brood year 1984 half of the egg lots tested positive for the presence of bacteria within the yolk of surface sterilized. unfertilized eggs, one fourth of these egg lots contained Listeria sp. 3nd the average mortality for 311 egg lots was approximately 17%. In the 1985 brood year, the number of egg lots containing bacteria was twice that of brood year

'84, the incidence of Listeria sp. was twice 3v and the average mortality for 311 egg lots was twice as high. Data from four brood years prior to initiation of this project show that 198-I egg lots experienced the lowest average mortality over six years while 1985 experienced the highest mortalities in the same time span. This shows that there are annual variations in both the severity of early life stage mortalities and the frequency of egglots contaminated with bacteria.

Efforts to quantitate the numbers of bacteria present within the yolk of freshly spawned eggs by plate count were equivocable. Some of the eggs tested sterile by direct plating but would yield 3 variety of species when tested following thioglycollate enrichment. Other egg lots yielded growth by direct plating but these proved to be mostly rapid growing Enterobacters, Moraxella etc. Following enrichment, some of these egg lots would yield additional species. The only Gram positive bacterium isolated regularly without prior enrichment was Staohvlococcus aureus. Three explanations exist for the apparent sterility of yolk when tested by direct plating.

1. The bacteria may be present in the yolk in such low numbers that they could be missed when aliquots of the sample are plated.
2. The bacteria may be of a type (eg. Listeria) which requires protracted cnrichmcnt before it is able to grow on plated media.
3. Some of the eggs in an egg lot may contain bacteria and others may not.

Enrichment of samples in thioglycollatc does not allow for meaningful quantitation of bacteria. however. it does allow for the qualitative detection of a wide variety of aerobic. microaerophilic and anaerobic organisms which may be present in very low numbers. Our histologic preparations of unfertilized eggs often require protracted periods of examination under oil immersion before detecting 3 bacterium. This supports our belief that low numbers of bacteria are in eggs at spawning. Similar examination of eggs which have been fertilized and incubntcd for several weeks reveal bacteria more rapidly since they arc there in greater numbers suggesting that the bacteria have been multiplying

within the egg during incubation.

The high incidence of Listeria sp. was surprising. We have found only one report (Stamatin et al., 1957) of this organism in fish. Botzler et al. (1973) reported the detection of this organism in frogs, turtles, leeches and snails. Weiss and Seeliger (1975) demonstrated that it was widespread in soil and on decaying vegetation. Listeriosis is generally considered to be a disease of individuals who have impaired immune systems. These individuals are most commonly the very young in which the immune system is not yet completely functional or older individuals whose immune system has begun to fail. O'Hara et al (1985) have demonstrated in the mouse newborn that there is a steady increase with age in the $LD_{,,}$ values for young mice challenged with Listeria (1 week $LD_{,,} = 4x10^{2}$, 4 week $LD_{,,} = 2.7x10^{5}$). They further showed that the immature macrophages, which in adults would normally phagocytose and lyse this intra-cellular bacterium, are not capable of lysing the Listeria that they injest and these injested bacteria multiply within the immature macrophage. Groves and Welshimer (1977) tested a group of 112 Listeria isolates (some of which were known pathogens since they were isolated from active cases of listeriosis, the other isolates were apparently apathogenic) for characteristics which could be associated with pathogenic strains. They found that 98% of the known pathogenic isolates were beta hemolytic while the apathogenic strains were not. Many of our Listeria isolates were beta hemolytic suggesting that they merit consideration as potential pathogens in young salmonids. The potential of the immature macrophage in developing salmonids in relationship to intracellular organisms such as Listeria sp. and Renibacterium salmoninarum need to be explored.

The detection of endotoxin in ovarian fluid and yolk is not surprising since a variety of Gram negative bacteria were isolated from these samples. Several review articles on the effect of endotoxins on physiologic and immune systems are: Osterud (1985), Bradley (1979) and Morrison and Ryan (1979). Reviews on the Shwartzman Reaction which can be induced by two injections of endotoxin 24 hours apart are by Hjort and

Rapaport (1963, Thomas and Good (1952) and Good and Thomas (1952). Histolgic evidence for a generalized Shwartzman Reaction are: intravascular clot formation with subsequent areas of necrosis, aggregation of neutrophils in capillary beds and damaged endothelial cells resulting in edema and leakage of erythrocytes into interstitial spaces. Hematologic findings would be: reduced neutrophil counts, increased hematocrit levels, and increased time for clot formation. Additionally, Thomas and Good (1952) report the occlusion of the capillary bed of the glomerulus with an amorphous unknown substance, which results in blockade of vascular service to the remainder of the nephric system which can cause damage to the epithelium of these tubules. Blockage of the glomerular capillary bed and tubule damage are commonly seen in the kidney samples we have studied. Areas of edema and damage to the capillary endothelium are seen with frequency in areas of the fins. Similarly, areas of intravascular clot formation are detected in the liver.

Depending on the species, bacteria produce other substances such as hemolysins, proteases and toxins. Wigney et al. (1978) has demonstrated that a combination of Aeromonas hydroohila endotoxin and hemolysin would induce Wed Leg Disease in frogs whereas neither of these bacterial products alone would induce the symptoms of the disease. Thune et al. (1982) has shown that both hemolysin and a heat stable protease from A. hydrophila are more lethal to catfish than a heat labile protease. Their conclusions were from using highly purified, heat treated extra cellular products. They further show that both the heat stable and heat labile proteases when injected into fish, without prior heat treatment of the proteases, were 100% lethal. This last test demonstrates that the use of heat in extraction and purification of bacterial extra cellular products can aslo destroy physiologic properties of the extract.

Wedemeyer and Ross (1968) studied the physiologic effect of purified endotoxin in salmon and trout over a 48 hour period. The A. safmonicida endotoxin used was extracted using hot phenol-water. They found accelerated liver glycogen depletion but no other liver or cardiovascular effects were detected. thune (1982b) reported no pathologic effects of

endotoxin in catfish. Patterson and Fryer (1973) also did not detect mortalities resulting from A. salmonicida endotoxin injection into young salmon (Oncorhvnchus kisutch). Thomas and Good (1952) demonstrated that the pathologic sequlae to endotoxin exposure could be induced in the Rabbit by two exposures to endotoxin 24 hours apart. Not until histopathologic examination of fish exposed several times to endotoxin are done can it be ascertained whether or not fish are susceptible to the toxic effects of endotoxins as demonstrated in mammals by several authors.

Our concern over endotoxins centers on its potential effect, as demonstrated in mammals, on the immune system. It is known that endotoxin causes a precipitous decline in the circulating neutrophils which may remain low for several days. The neutrophil is the primary responder in the leucocyte series to foreign antigen and depression of these cells renders the animal less able to resist infection. Endotoxin also increases the activity level of the other phagocytic cells some of which may not be able to lyse substances such as bacteria and viruses. Under these circumstances pathogens which can live within cells such 3s Listeria and viruses can establish themselves and live sequestered from the effects of the immune system when it returns to normal. Gut (1982) demonstrated in the rat that surgical removal of the colon (the source of endotoxins produced by the intestinal flora) protected these animals from 100% lethal doses of Frog Virus 3 Hepatitus. This same group of animals experienced 89% mortality after injection of 0.01 of a 100% lethal dose of endotoxin. They conclude that endotoxin plays a role in the pathogenesis of this virus. We have found a wide range of endotoxin in ovarian fluid, yolk of incubating eggs and artificial diets fed to the fish. Challenges need to be conducted with fish viral pathogens and intracellular bacteria to determine if the $LD_{,,}$ values are different for groups of fish which have been given endotoxin prior to, following, and during challenge and these would be compared with challenge groups which received no endotoxin. Ideally, the endotoxin used should be 3 cold extract which would more nearly mimic the "native" state rather than a hot phenol-water extract.

Acknowledgments

We wish to thank the Bonneville Power Administration for their financial support of this project, also J. L. Banks, D. A. Leith, L. G. Fowler, J. Morrison and the entire staff of Abernathy Salmon Culture Technology Center for their time and effort expended in our behalf. Further thanks go to J. W. Rourke and the staff of the Clinical Microbiology section for allowing us use of their equipment, Mary Jo Florey of the Red Cross Blood Bank for similar courtesies, Dr. J. Crosa, OHSU Department of Microbiology for providing G + C ratios on our Vibrio isolates, Genevieve Nygaard of the California Department of Health Services for her recent information on identification of various Listeria species and to the National Veterinary Services Laboratories for providing confirmation of identification of some of our isolates. Last, but not least, we thank Lanette McGowan for her cheerful performance in preparing this report.

References

References

Agersborg, H. P., 1933. Salient Problems in the Artificial Rearing of Salmonoid Fishes, With Special Reference to Intestinal Fungisitosis and the Cause of White-spot Disease. Trans. Amer. Fish. Soc. 63:240-250.

Banks, J. L., 1983. Artificial Substrate in Vertical Incubators: A Method for Controlling Coagulated Yolk Disease in Chinook Salmon, Oncorhynchus tshawytscha (Walbaum). Technology Transfer Series No. 83-1.

Bergey, D. H., 1974. Bergey's Manuai of Determinative Bacteriology. Buchanan, R. E. and Gibbons, N. E. eds. Williams and Wilkins Co., Baltimore.

Botzler, R. G., Wetzler, T. F. and Cowan, A. B., 1973. Listeria in Aquatic Animals. J. Wildlife Dis. 9: 163- 170.

Bradley, S. G., 1979. Cellular and Molecular Mechanisms of Action of Bacterial Endotoxins. Ann. Rev. Microbial., 33:67-94.

Bullock, G. L., Stuckey, H. M. and Mulcahy, D., 1978. Corynebacterial Kidney Disease: Egg Transmission Following Iodophore Disinfection. Fish Health News 7:51-52.

Cone, D. K., 1982. A Lactobacilltq sp. from Diseased Female Rainbow Trout, Salmo gairdneri Richardson, in Newfoundland, Canada. J. Fish Diseases 5:479-485.

Evelyn, T. P. T., Ketcheson, J. E. and Prosperi-Porta, L., 1984. Further Evidence for the Presence of Renibacterium salmoninarum in Salmonid Eggs and for the Failure of Povidone-Iodine to Reduce the Intra-Oyum Infection Rate in Water-Hardened Eggs. J. Fish Diseases 7:173-182.

Fowler, L. G., 1981. Protein and energy relation of starting diets for Chinook salmon fry. Prowess in Fish Culture 43:15 1-1 53.

Fowler, L. G., 1980. Substitution of soybean and cottonseed products for fish meal in diets fed to Chinook and Coho salmon. Progress in Fish Culture 42:87-91.

Good, R. A. and Thomas, L., 1952. Studies on the Generalized Shwartzman Reaction. II. The Production of Bilateral Cortical Necrosis of the Kidneys by a Single Injection of

Bacterial Toxin in Rabbits Previously Treated With Thorotrast or Trypan Blue. J. Exptl, Med. 96:625-64 1.

Groves, R. D. and Welshimer, H. J., 1977. Separation of Pathogenic from Apathogenic Listeria Monocytogenes by Three in Vitro Reactions. J. Clin. Microbial. 5:559-563.

Gulberlet, J. E., Samson, V. J. and Brown, W. H., 1931. A Report of Hydeocoele Embryonalis, or Yolk-Sac Disease, in Salmon Fry, With a Note on its Cause. Trans. Am. Microscipial Soc. L:164-167.

Gut, J. P., Schmitt, S., Bingen, A., Anton, M and Kirn, A., 1982. Protective Effect of Colectomy in Frog Virus 3 Hepatitis of Rats: Possible Role of Endotoxin. J. Infect. Diseases 14635949605.

Hjort, P. F. and Rapaport, S. I., 1965. The Shwartzman Reaction: Pathogenetic Mechanisms and Clinical Manifestations. Ann, Rev. Med. 16:135-168.

Jay, J. M., Margitic, S., Shereda, A. L. and Covington, H. V., 1979. Determining Endotoxin Content of Ground Beef by the Limulus Amoebocyte Lysate Test as a Rapid Indicator of Microbial Quality. Appl. Env. Microbial. 38:885-890.

Leach, G. C,, 1924. Artificial Propagation of Brook Trout and Rainbow Trout, With Notes on Three Other Species. Wpt. U. S. Comm. of Fish for 1923, Appendix VI, Doc. 955, pp. 1-74.

Levin, J., Tomasulo, P. A. and Oser, R. S., 1970. Detection of Endotoxin in Human Blood and Demonstration of an Inhibitor. .I. Lab, Clin. 75:903-911.

Levin, J. and Bang, F. B., 1968. Clottable Protein in Limulus: Its Localization and Kinetics of its Coagulation by Endotoxin. Thromb. Diath. Haemorrh. 19:186-197.

MacKinnon, D., 1969. Effect of Mineral Enrichment on the Incidence of White-Spot Disease. Prog. Fish. Cult, 32:74-78.

MazuranichJ. J, and Nielson, W. E., 1959. White-Spot Disease of Salmon Fry. Prog. Fish. Cult,. 21(4):172-176.

Morrison, D. C, and Ryan, J. L., 1979. Bacterial Endotoxins and Host Immune Responses.

Advances in Immunology 28:293-449.

O'Hara, R., Mitsuyama, M., Masakazu, M. and Nomoto, K., 1985. Ontogeny of Macrophage-mediated Protection Against Listeria monocytoaenes. Infection and Immunity 48:763-168.

Osterud, B., 1985. Interaction of Endotoxins, Blood Elements and the Vessel Wall. Progress in Clinical and Biological Research 189:67-78.

Paterson, W. D. and Fryer, J. L., 1974. Effect of Temperature and Antigen Dose on the Antibody Response of Juvenile Coho Salmon (Oncorhvnchus kisutch) to Aeromonas salmonicida Endotoxin. J. Fish Res. Bd. Canada 3 1:1743-1749.

Rigney, M. M., Zilinsky, J. W. and Rouf, M.A., 1978. Pathogenicity of Aeromonas hydrophila in Red Leg Disease in Frogs. Current Microbiology 1:175-l 79.

Stamatin, N. C., Ungureanu, C., Constantinescu, E., Solnitzky, A. and Vasilescu, E., 1957. Infectia naturala cu Listeria monocytoeenes la pastravul curcubeu Salmo irideus. Annals of the Institute of Animal Patholoev and Hveiene (Bucharest) 7:163-180.

Sullivan, J. D., Ellis, P. C., Lee, R. G., Combs. W. S. and Watson, S. W., 1983. Comparison of the Limbus Amoebocyte Lysate Test With Plate Counts and Chemical Analyses for Assessment of the Quality of Lean Fish. Appl. Env. Microbial. 45:720-722.

Thomas, L. and Good, R. A., 1952. Studies on the Generalized Shwartzman Reaction. I. General Observations Concerning the Phenomenon. J. Exptl. Med. 96:605-623

Thune, R. L., Graham, T. E., Riddle, L. M. and Amborski, R. L., 1982. Extracellular Products and Endotoxin from Aeromonas hvdrophila: Effects on Age-0 Catfish. Trans. Am. Fish. Soc. 111:403-408.

Thune, R. L., Graham, T. E., Riddle. L. M. and Xmborski, R. L., 1982b. Extracelular Proteases from Aeromonas hydeophila: Partial Purification and Effects on Age-0 Channel Catfish. Trans. Am. Fish. Soc. 111:749-754.

Von Betegh, L., 1912. Hydroccle Embryonalis. Central b. f. Bakt., Parsit. and Infckt. Abt. 1, 66:284-286. English translation, Anne L. Masey, Scientific Investigations. 1913 So.

3. Dept. .Agr. & Techn. Instruction for Ireland Fisheries Branch.

Wedemeyer. G.. Ross. A. J., 1969. Some Metabolic Effects of Bacterial Endotoxins in Salmonid Fishes. J. Fish Rcs. Bd. Canada 26:115-121.

Weis. J. and Secliger, H. P. R., 1975. Incidence of Listeria monocytoeenes in Nature. Applied Microbiology 30:29-32.

Wolf K.. 1970. White-Spot Disease of Fish Eggs and Fry. FDL-22, Bureau of Sport Fisheries and Wildlife, Washington, DC.

Wood, J. W., 1974. Diseases of Pacific Salmon, Their Prevention and Treatment. 2nd Ed. Washington State Department of Fisheries, Olympia, WN.

Tables

Table1

BACTERIA ISOLATED FROM YOLK OF EGGS, OVARIAN FLUID AND MATERNAL BLOOD OF SIXTY FALL CHINOOKSALMON

Genus	Species	Number of samples from which bacterial species were isolated: Egg yolk	Adult blood	Ovarian fluid	Ovarian fluid and yolk	Total No. of isolations
Acinitobacter	anitratus	1	1			2
	sp.	1				1
Aerococcus	viridans		1		1	2
Aeromonas	hydrophila	20	18	10	14	62
	salmonicida	2				2
	shigelloides				1	1
Alcaligenes	sp.	4				4
Bacillus	sp.	5				5
Citrobacter						1
Corynebacterium						
	hoffmanii	6		1		7
	sp.			1		1
Enterobacter	aerogenes	2				2
	agglomerans	1			2	5
	cloacae	2				4
	sakazaki				4	4
	SP.				1	1
Escherichia	coli				1	1
Hafnia	alvei	2			4	7
Listeria	sp.	12		1	18	35
Macrococcus		3				3
Moraxella	osloensis	1			4	5
	sp.	2				2
Pseudomonas						
	aeruginosa	2				2
	cepacia					1
	fluorescens	6	2			16
	pancimobilis	1				2
	putida			3	3	6
	putrefaciens			4	3	8
	stutzeri	3				5
	sp.	9			4	13
Pasteurella	multocida					1
	SP.	1				2
Peptostreptococcus		1				1
Serratia	liquefaciens	3				4
	marcescens					3
	sp.				3	3
Staphylococcus	aureus	15				15
	epidermidis	2				2
Streptococcus						
	a hemolytic	1				2
	B hemolytic	1				1
Vibrio	extorquens	1				1
	fluvialis	5				6
	parahemolyticus	5				5
	sp.	1				1
Yersinia	enterocolitica	1				2
	ruckeri	2				2

Table 2

INCIDENCE OF BACTERIAL GENERA ISOLATED FROM WITHIN SURFACE STERILIZED, UNFERTILIZED EGGS OF SALMON (Oncorhynchus tshawytscha) LISTED IN ORDER OF FREQUENCY OF ISOLATIONS.[1]

Genera	Egg Lots Found In	Species
Aeromonas sp.	22	(20 hydrophila, 2 salmonicida)
Pseudomonas sp.	21	(6 fluorescens, 3 stutzeri, 2 aeruginosa, 1 pancimobilis, 9 unspeciated)
Staphylococcus sp.	17	(15 aureus, 2 epidermidis)
Vibrio sp.	12	(5 parahemolyticus, 5 fluvialis, 1 extorquens, 1 unspeciated)
Listeria sp.[2]	12	
Corynebacterium sp.	6	(6 hoffmanii)
Bacillus sp.	5	
Enterobacter sp.	5	(2 aerogenes, 2 cloacae, 1 agglomerans)
Alcaligenes sp.	4	
Macrococcus sp.	3	
Moraxella sp.	3	
Serratia sp.	3	
Yersinia sp.	3	(2 ruckeri, 1 enterocolitica)
Acinetobacter sp.	2	(1 anitratus, 1 unspeciated)
Streotococcus sp.	2	(1 alpha, 1 beta hemolytic)
Hafnia sp.	2	
Pasteurella sp.	1	
Peptostreotococcus	1	

Summary: 124 isolates were established from within the yolk of surface sterilized eggs. Within this group of isolates were found 18 different genera containing representatives of 32 different species.

1 Based on the study of eggs from 60 salmon (30 each in brood years '84 and '85)

2 This number may be artificially low in sterilized eggs since 24 isolations were made in unsterilized eggs coated with ovarian fluid whereas ovarian fluid alone yielded only one isolate of Listeria sp.

Table 3

BACTERIA ISOLATED FROM THE SURFACE OF INCUBATING SALMON EGGS WHICH EXHIBITED SOFT SHELL DISEASE

Isolated only from Abernathy SCTC samples	Isolate from both Abernathy SCTC and Spring Creek NFH samples	Isolated only from Spring Creek NFH Samples
Vibrio anguillarum	*Aeromonas hydrophila*	*Pseudomonas fluorescens*
Pseudomonas maltophilia	*Enterobacter agglomerans*	" *putida*
" *pancimobilis*	*Klebsiella pneumonae*	*Chromobacter* Sp.
Corynebacterium Sp.		
Shigella dysenteriae		
Staphylococcus aureus		

FIGURES

FIGURE 1

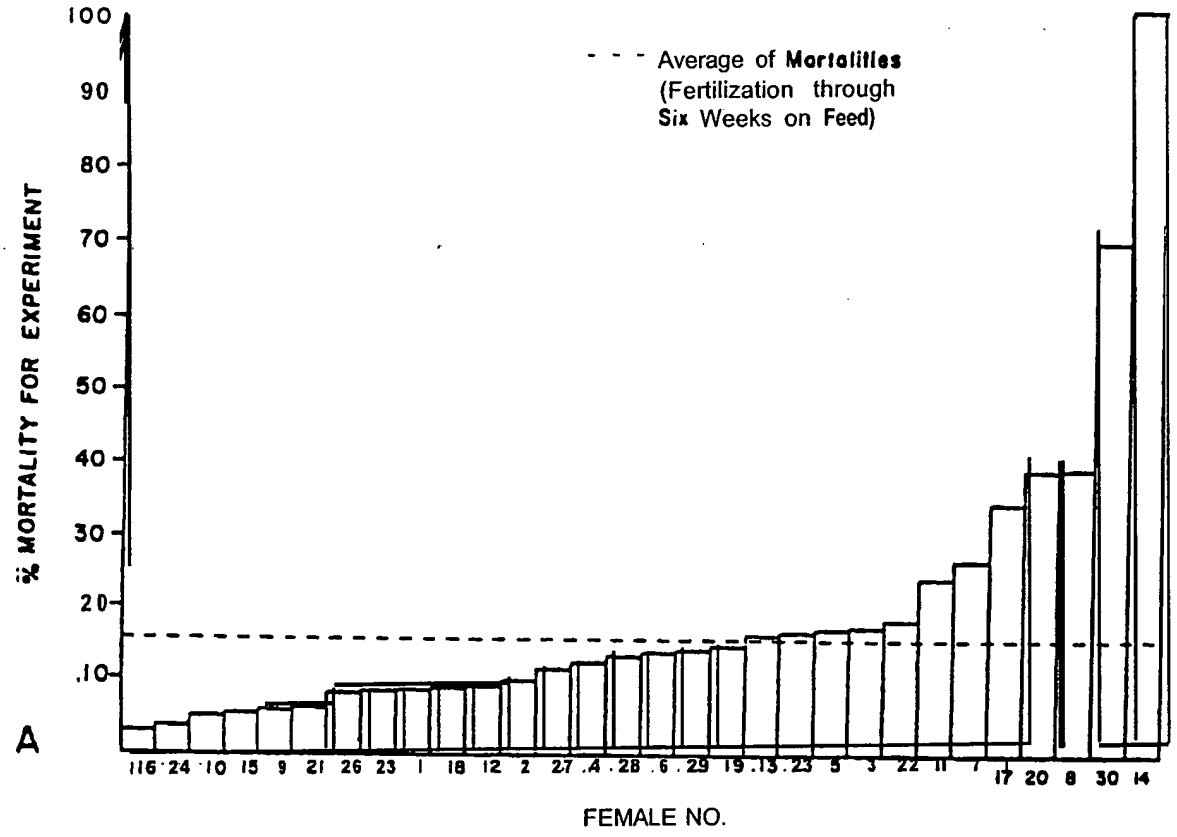
PROCEDURE FOR OBTAINING AND IDENTIFYING MICROORGANISMS FROM CHINOOK SPECIMENS

Aseptically Obtained Specimen:	Preliminary Inoculation Medium, incubation conditions[1]	Growth from Preliminary Medium Transferred to:	Organisms isolated at left identified by:
0.5ml blood	FTG', **6°C,** 7 days	Aerobic: Blood agar, **6°C,** 7 days. Brucella agar, **6°** 7 days. Anaerobic: Blood agar, MacConkey agar, **6°C,** 7 days. **CO_2:** Chocolate agar, **6°C,** 7 days	Gram stain, morphology, biochemical reactions (Bergey's Manual), API system. Drug sensitivities determined for each isolate
0.5 ml ovarian fluid	FTG, **6°C,** 35 days	As above	As above
	Aerobic: blood culture medium, **6°C,** 14 days	As above	As above
	Anaerobic: blood culture medium, **6°C,** 14 clays	As above	As above
Salmon **eggs**[2]	FTG, **6°C,** 14 days	As above	As above
	FTG, **18°C,** 14 days	As above	As above

1 FTG = Fluid thioglycollate medium.

2 Where organisms are sought inside of eggs, eggs are first surface sterilized before being placed, intact, in FTG. Eggs in those FTG tubes which showed no growth after 7 days were crushed in situ and tube incubated at the same temperature for 28-35 days.

Fig. 2


100
90
80
70
60
50
40
30
20
10
% MORTALITY FOR EXPERIMENT
Average of Mortalities
(Fertilization through
Six Weeks on Feed)
A
FEMALE NO.

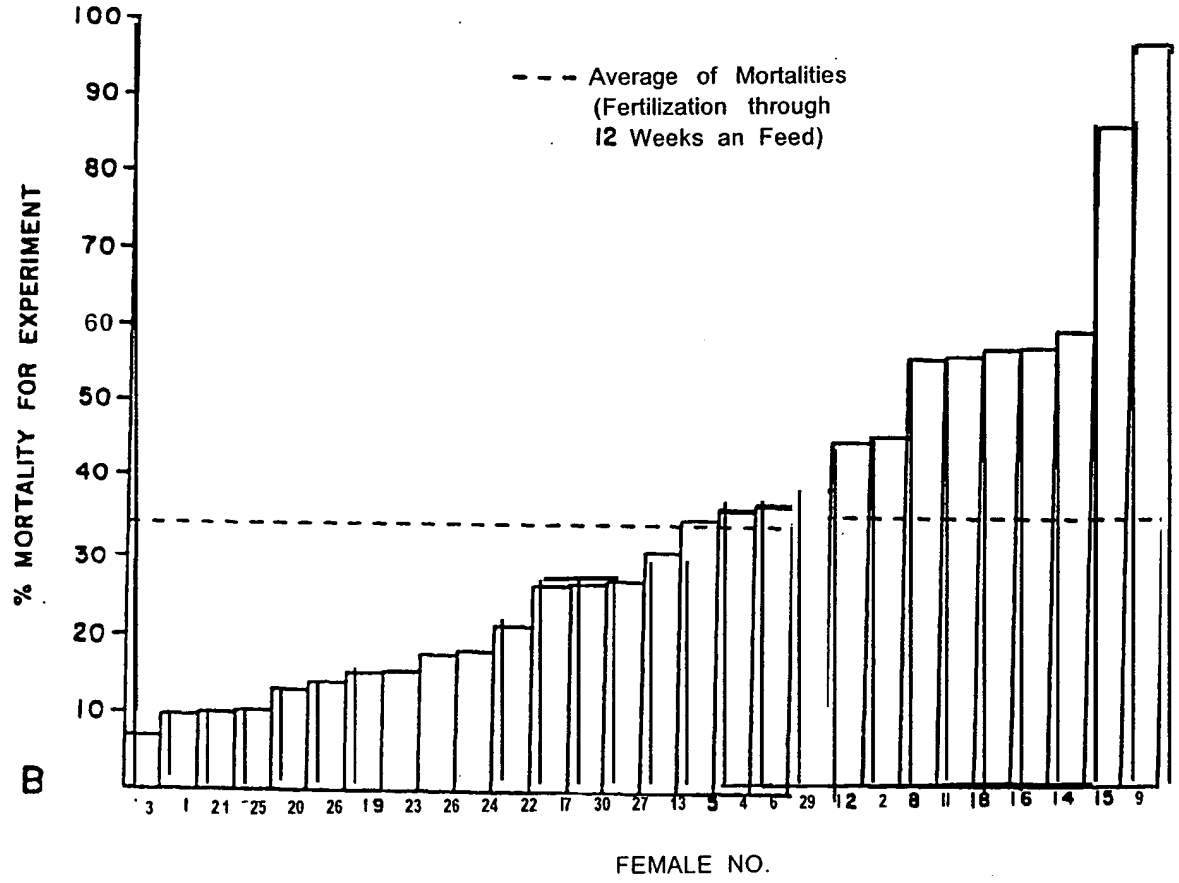
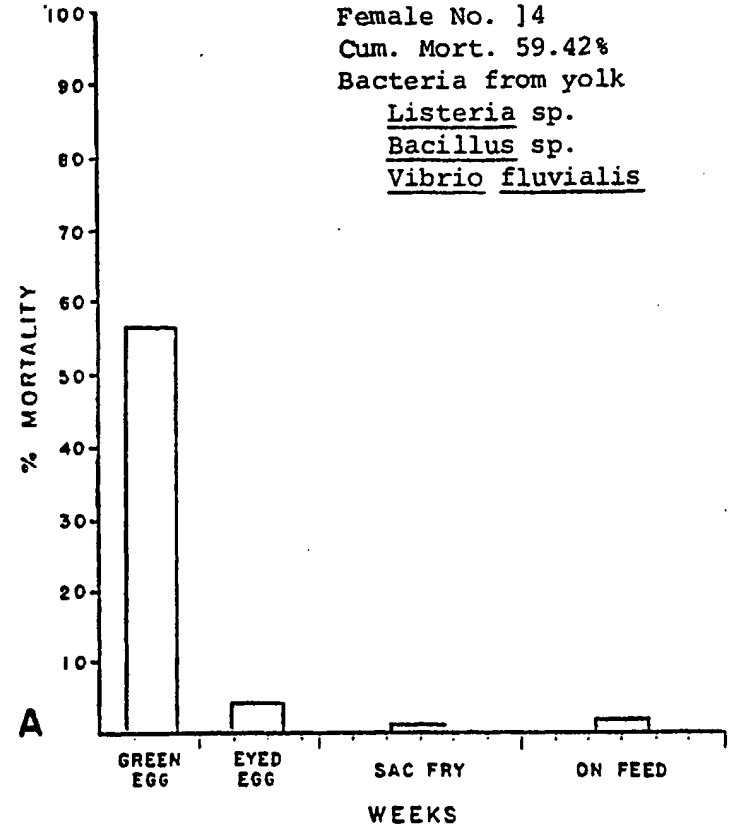

100
90
80
70
60
50
40
30
20
10
% MORTALITY FOR EXPERIMENT
Average of Mortalities
(Fertilization through
12 Weeks an Feed)
B
FEMALE NO.

Fig. 3


Female No. 14
Cum. Mort. 59.42%
Bacteria from yolk
Listeria sp.
Bacillus sp.
Vibrio fluvialis
% MORTALITY
100
90
80
70
60
50
40
30
20
10
A
GREEN EGG
EYED EGG
SAC FRY
ON FEED
WEEKS

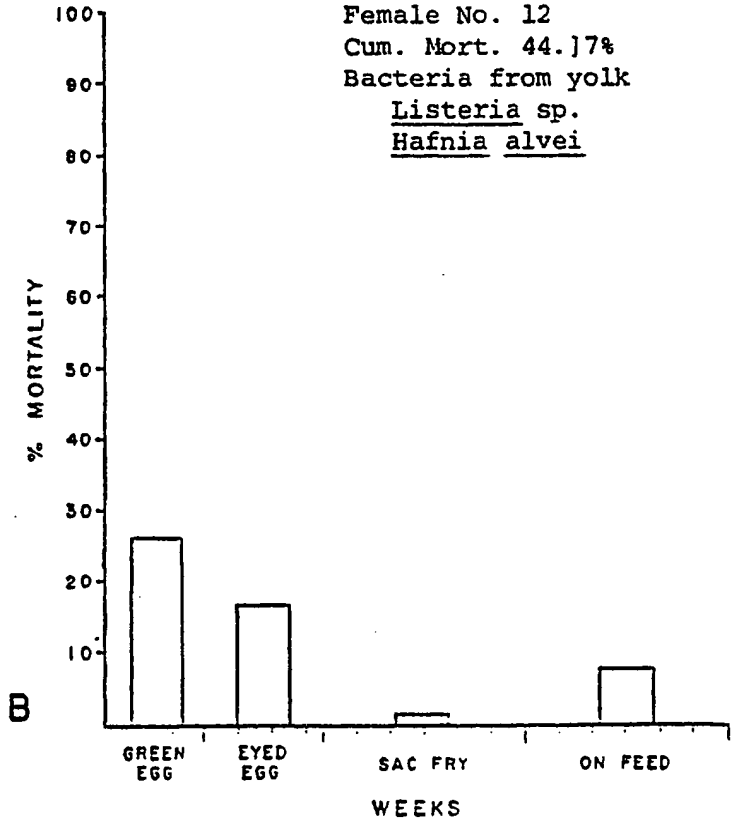
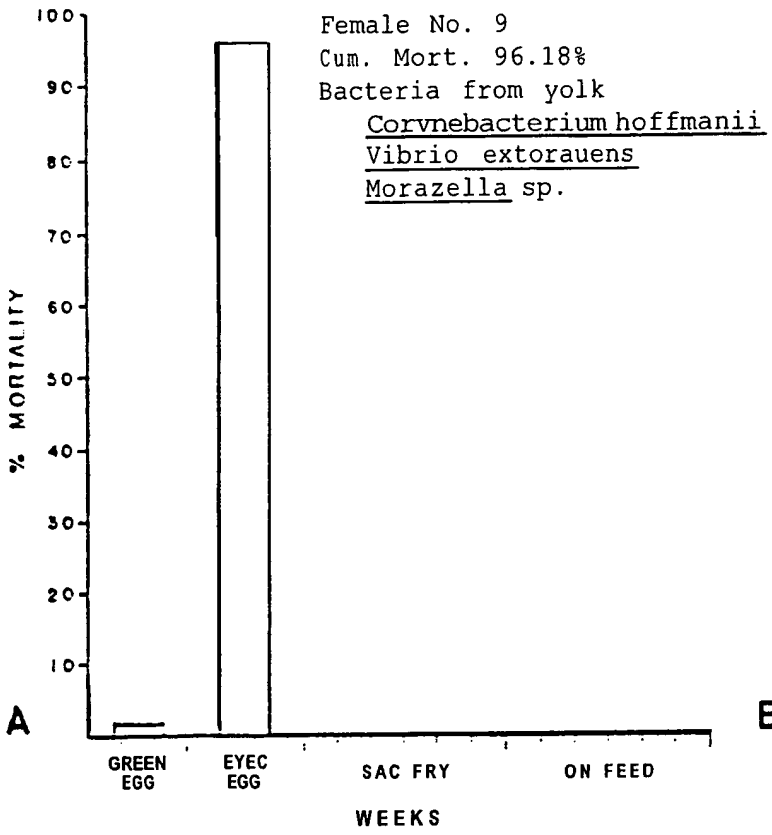
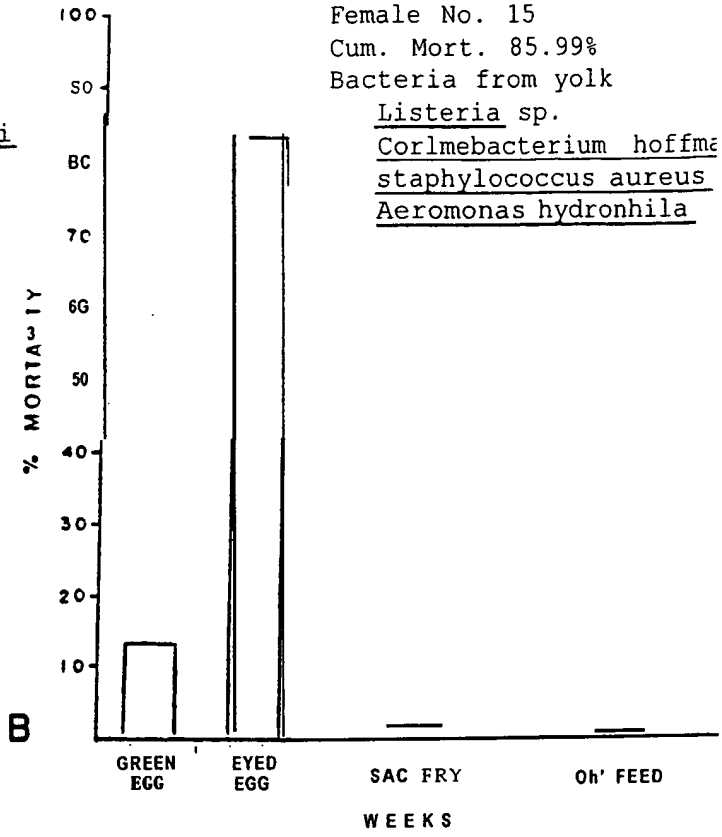
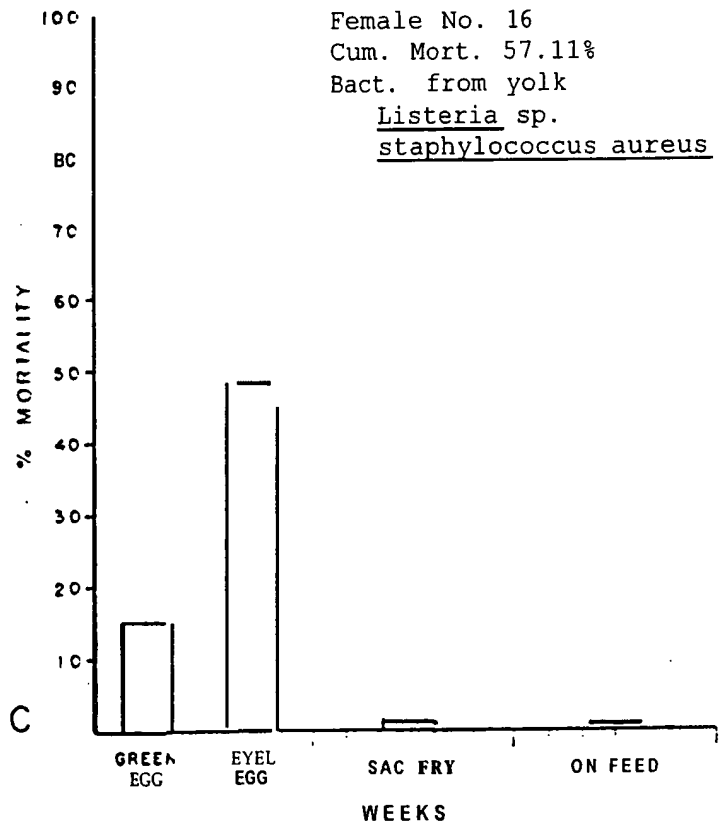

Female No. 12
Cum. Mort. 44.17%
Bacteria from yolk
Listeria sp.
Hafnia alvei
% MORTALITY
100
90
80
70
60
50
40
30
20
10
B
GREEN EGG
EYED EGG
SAC FRY
ON FEED
WEEKS

Fig. 4 .


Female No. 9
Cum. Mort. 96.18%
Bacteria from yolk
Corvnebacterium hoffmanii
Vibrio extorauens
Morazella sp.
% MORTALITY
100
90
80
70
60
50
40
30
20
10
A
GREEN EGG
EYEC EGG
SAC FRY
ON FEED
WEEKS


Female No. 15
Cum. Mort. 85.99%
Bacteria from yolk
Listeria sp.
Corlmebacterium hoffma
staphylococcus aureus
Aeromonas hydronhila
% MORTALITY
100
90
80
70
60
50
40
30
20
10
B
GREEN EGG
EYED EGG
SAC FRY
Oh' FEED
WEEKS


Female No. 16
Cum. Mort. 57.11%
Bact. from yolk
Listeria sp.
staphylococcus aureus
% MORTALITY
100
90
80
70
60
50
40
30
20
10
C
GREEN EGG
EYEL EGG
SAC FRY
ON FEED
WEEKS

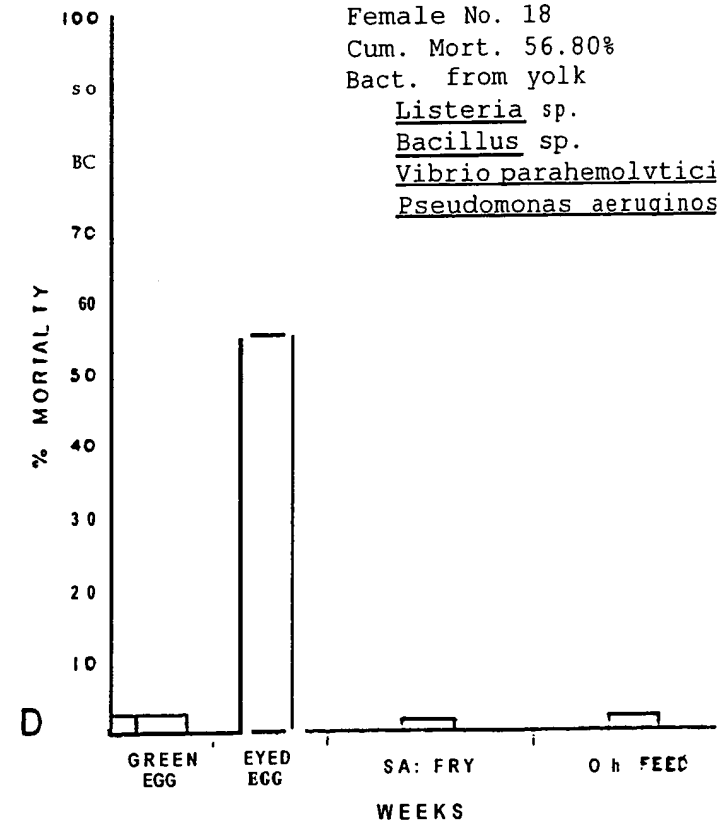
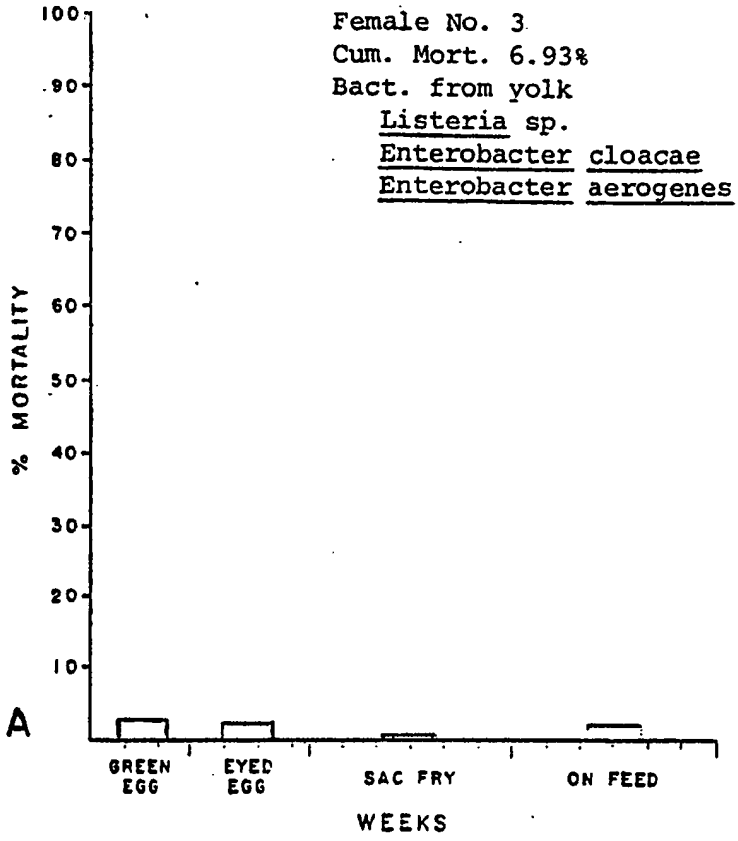

Female No. 18
Cum. Mort. 56.80%
Bact. from yolk
Listeria sp.
Bacillus sp.
Vibrio parahemolvtici
Pseudomonas aeruginos
% MORTALITY
100
90
80
70
60
50
40
30
20
10
D
GREEN EGG
EYED EGG
SA: FRY
O h FEED
WEEKS

Fig. 5


Female No. 3
Cum. Mort. 6.93%
Bact. from yolk
Listeria sp.
Enterobacter cloacae
Enterobacter aerogenes
% MORTALITY
100
90
80
70
60
50
40
30
20
10
A
GREEN EGG
EYED EGG
SAC FRY
ON FEED
WEEKS

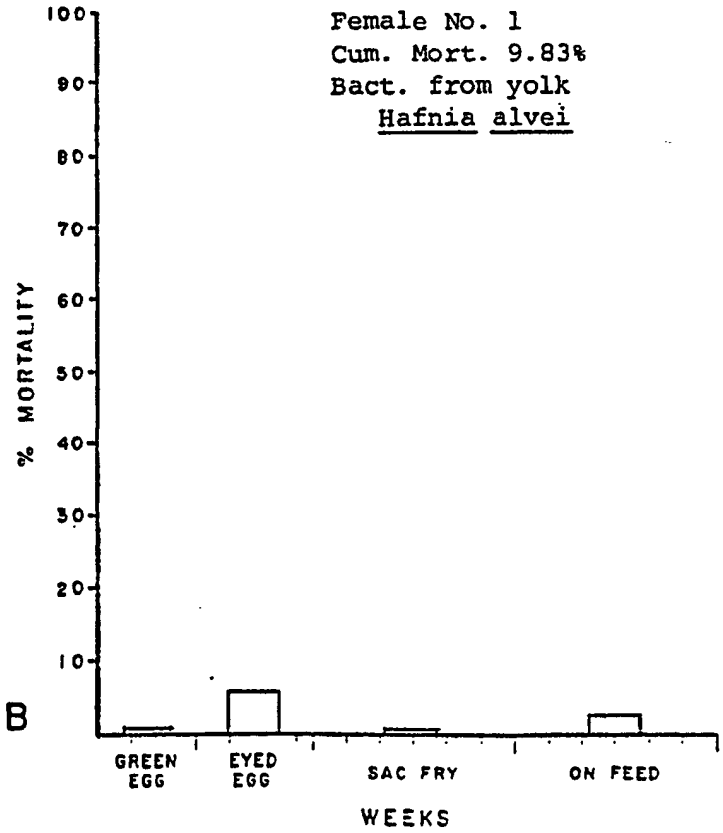

Female No. 1
Cum. Mort. 9.83%
Bact. from yolk
Hafnia alvei
% MORTALITY
100
90
80
70
60
50
40
30
20
10
B
GREEN EGG
EYED EGG
SAC FRY
ON FEED
WEEKS

Figure 6
EGG LOTS WITH LOWEST CUMULATIVE MORTALITY FROM FERTILIZATION TO SWIM-UP IN BROOD YEAR 1985

Spec. #	Cum. Mort.	Yolk Unenriched Blood Agar	Yolk Unenriched Cytophaga Media	Yolk Enriched Blood Agar
3	5.03%	NG	NG	70,000
1	7.36%	NG	NG	30,000
25	8.21%	20,000	NG	5,000
20	11.45%	30,000	NG	>100,000
28	13.24%	30,000	NG	10,000
24	13.25%	20,000	NG	20,000

EGG LOTS WITH HIGHEST CUMULATIVE MORTALITY FROM FERTILIZATION TO SWIM-UP IN BROOD YEAR 1985

Spec. #	Cum Mort.	Yolk Unenriched Blood Agar	Yolk Unenriched Cytophaga Media	Yolk Enriched Blood Agar
9	96.18%	10,000	NG	30,000
15	85.84%	NG	NG	>100,000
14	58.73%	NG	NG	80,000
16	56.82%	NG	NG	80,000
18	56.07%	20,000	NG	>100,000
11	54.83%	NG	NG	>100,000

Figure7
MICROORGANISMS RECOVERED BY "ENRICHMENT" METHOD FROM SURFACE DISINFECTED NONFERTILIZED EGGS OF SALMON WHOSE OFFSPRING EXPERIENCED HIGH MORTALITY RATES FROM FERTILIZATION THROUGH 12 WEEKS ON FEED.

Brood year 1984

Eggs from Salmon No.	Gram Positive Bacterial species	Gram Negative Bacterial species	% Cumulative Mortality
14	Listeria sp.	Aeromonas hydronhila Pseudomonas fluorescens putrefaciens Serratia sp.	100
30	Listeria sp.	Aeromonas hydrophila Enterobacter aglomerans Pseudomonas putrefaciens Serratia sp.	70.5
8	Listeria sp.	Pseudomonas fluorescens stutzeri Serratia sp.	39.36
20	Listeria sp.	none isolated	39.21
17	None isolated	Aeromonas hydroohila Pseudomonas putida	35.04
7	None isolated	Aeromonas hydronhila	26.30

Brood year 1985

9	Corynebacterium hoffmanii	Vibrio extorquens, Moraxella sp.	96.2
15	Listeria sp., corynebacterium **hoffmanii**, Staphy-**lococcus**, aureus	Aeromonas hydroohila	86.0
14	Listeria sp., Bacillus sp.	Vibrio fluvialis	59.4
16.	Listeria sp., Staphylococcus	None isolated	57.1
18	Listeria sp., Bacillus sp.	Vibrio parahemolyticus Pseudomonas aeruginosa	56.8
11	Listeria sp., Corynebacterium hoffmanii, Staphylococcus epidermidis	None isolated	55.7

Figure 8
MICROORGANISMS RECOVERED BY "ENRICHMENT" METHOD FROM SURFACE DISINFECTED NONFERTILIZED EGGS OF SALMON WHOSE OFFSPRING EXPERIENCED LOW MORTALLITY RATES FROM FERTILIZATION THROUGH 12 WEEKS ON FEED

Brood vear 1984

Eggs from Salmon No.	Gram Positive Bacterial species	Gram Negative Bacterial species	% Cumulative Mortality
16	None isolated	Yersinia enterocolitica	2.85
24	None isolated	Pseudomonas putrefaciens Hafnia alvei	3.84
10	None isolated	Aeromonas hydrophila Pseudomonas putida	5.25
15	None isolated	Pseudomonas sp.	5.54
9	None isolated	Pseudomonas putrefaciens	5.75
21	Listeria sp.	Aeromonas hydrophila Yersinia ruckeri	6.18

Brood Year 1985

3	Listeria sp.	Enterobacter cloacae, Enterobacter aerogenes	6.93
1	None isolated	Hafnia alvei	9.83
21	Listeria sp.	None isolated	10.43
25	Staphylococcus aureus	None isolated	10.66
20	Aerococcus viridans	Serratia liquefaciens, Enterobacter agglomerans, Aeromonas hydrophila	13.22
28	None isolated	Aeromonas hydrophila	13.82

Figure9.
NUMBER OF EGGLOTS FOUND TO CONTAINBACTERIAL GENERA: A COMPARISON OF THE 12 HIGHEST AND 12 LOWEST MORTALITY EGG LOTS OBSERVED IN 2 BROODYEARS.

No. of Egg Lots Found to Contain Bacterial Genera

Bacterial Genera	12 High Mortality Egg lots	12 Low Mortality Egg lots
Gram Positive		
Listeria	9	3
Corynebacterium	3	0
Staphylococcus	3	1
Bacillus	2	0
Aerococcus	0	1
Total Gram Positive	17	5
Gram Nesative		
Pseudomonas	7	4
Aeromonas	5	4
Serratia	3	1
Vibrio	3	0
Enterobacter	1	3
Moraxella	1	0
Hafnia	0	2
Yersinia	0	2
Total Gram Negative	20	16
Total all Isolations	37	21
Total # of Genera	10	9

Fig. 10

AVERAGE OF MORTALITIES EXPERIENCED BY EGG LOTS CONTAINING BACTERIA WITH DIFFERENT HEMOLYTIC CHARACTERISTICS

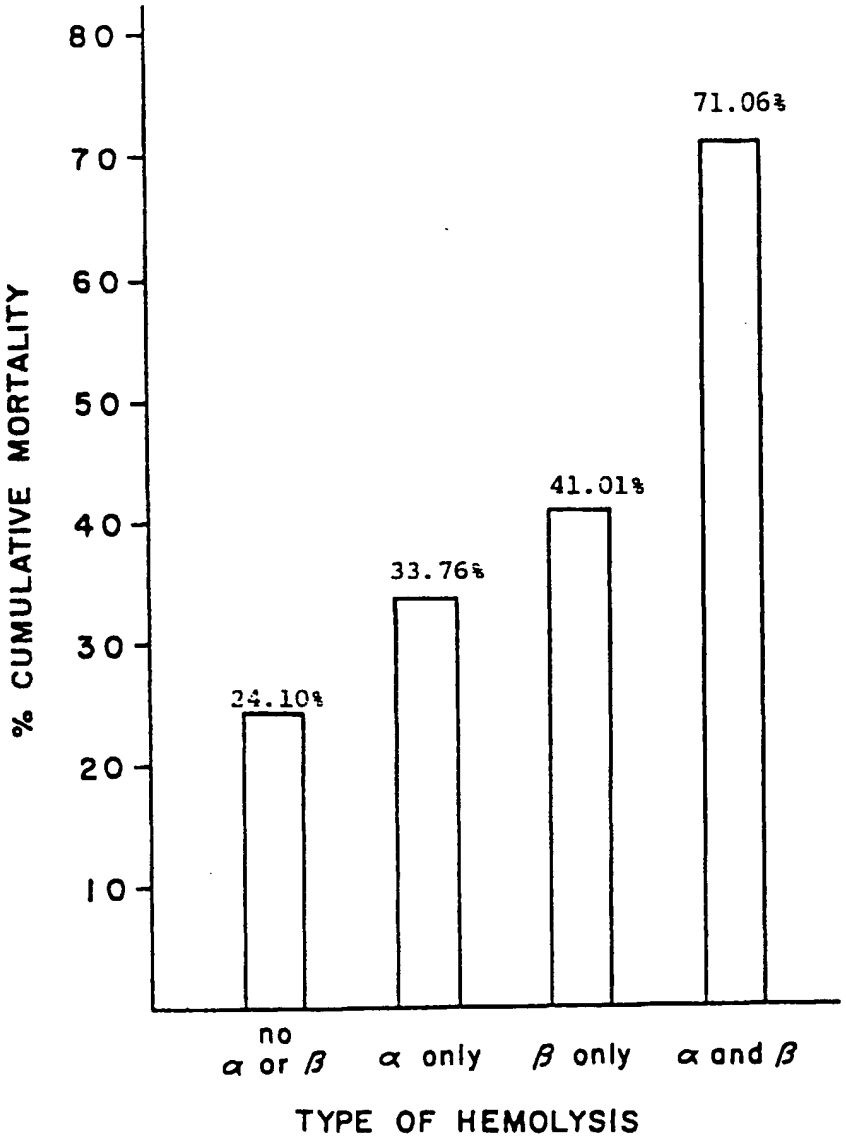
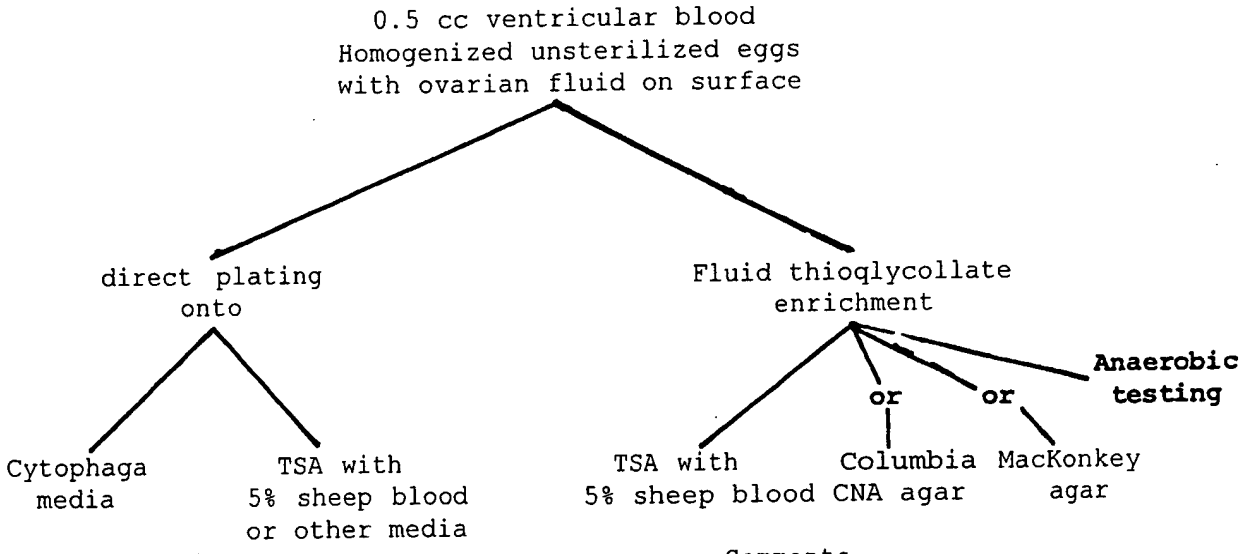


Figure 11
Proposed composite samples for capture of the majority of varieties of bacteria present within adult brood salmon



Comments	Comments
Allows quantiation	Quantiation not possible
Will isolate mostly rapid growing bacteria	Will isolate the more slow growing bacteria and those which are present in low numbers
Likely to give false test for sterility of sample	More accurate test for presence or absence of bacteria in sample
Will not detect anaerobes	Will allow detection of anaerobes

Figure 12
RELATIONSHIP OF BACTERIAL COLONY COUNTS TO ENDOTOXIN LEVELS AND GREEN EGG MORTALITY RATES IN 27 EGG LOTS SAMPLED AT SPAWNING. BACTERIAL COUNTS BY DIRECT PLATING WITHOUT PRIOR ENRICHMENT.

Plate Counts From Yolk (Bacteria/cc Yolk)	Average Endotoxin Units Per Egg[1]	Average Green Egg Mortality[2]	Range of Endotoxin Levels	Comments
No Growth	0.152 EU	5.22%	0.0-0.35 EU	1 of 11 contain Listeria
1-10,000	0.31 EU	3.07%	0.15-0.46 EU	3 of 3 contain Listeria
10,000- 100,000	0.399 EU	10.73%	0.18-0.81 EU	11 of 13 contain Listeria

[1]Average green egg mortality in 27 egg lots = 8.22%
[2]Average endotoxin/egg in 27 egg lots = 0.290 EU

Fig. 13

AVERAGE ENDOTOXIN LEVELS IN YOLK OF UNFERTILIZED EGGS AND SUBSEQUENT GREEN EGG MORTALITIES IN 30 EGG LOTS (FALL CHINOOK SALMON, Oncorhynchus tshawytscha)

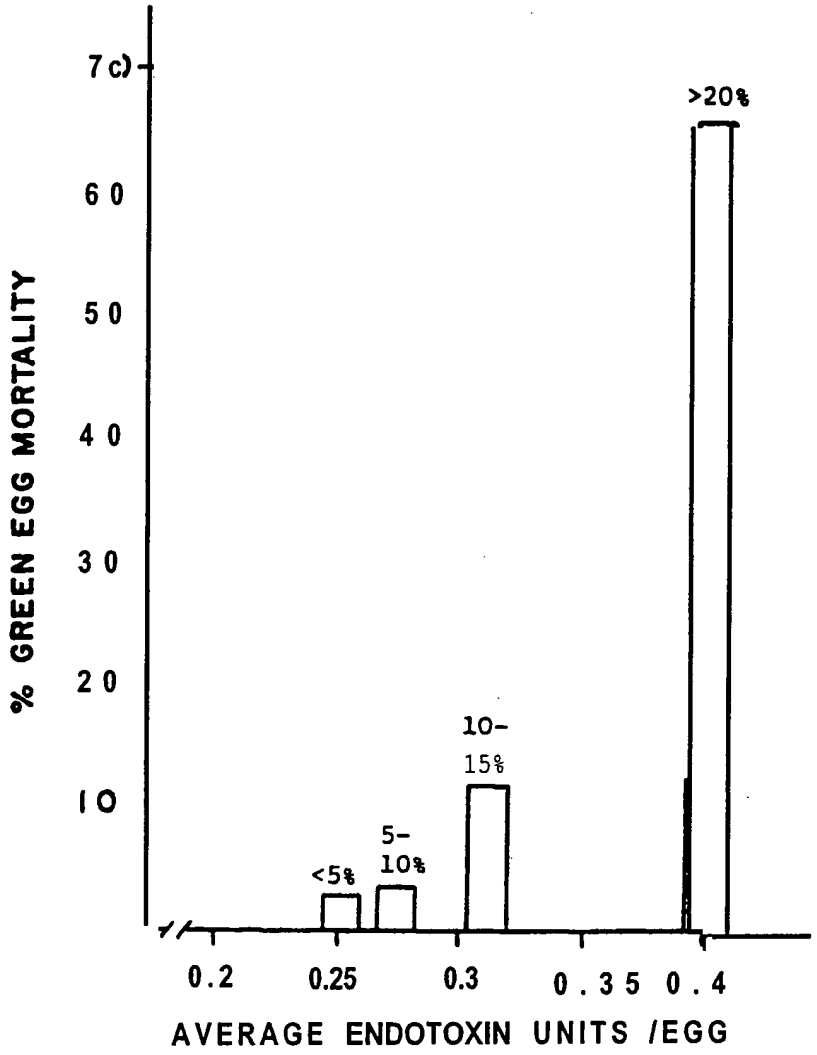


Figure 14
ENDOTOXIN DETERMINATIONS

ENDOTOXIN LEVELS IN YOLK OF UNFERTILIZED EGGS COLLECTED AT SPAWNING GROUPED ACCORDING To PRESENCE OR ABSENCE OF *LISTERIA* SP.

	EU*/egg	EU Range	Total No. Egg Lots	Average GE Mortality
Average all Egg Lots'	0.288	0.0-0.81	27	7.72%
Average all Egg Lots Without *Listeria* Sp.	0.194	0.0-0.38	13	4.31%
Average all Egg Lots With *Listeria* Sp.	0.375	0.05-0.81	14	10.89

EU=Endotoxin Unit-0.1 ng Endotoxin
'Based on 27 Egg lots

Figure 15
PERCENT MORTALITY AND VARIATION IN LENGTH AND WEIGHT GAIN COMPARED WITH CONTROLS IN GROUPS OF FISH CHALLENGED WITH BACTERIA AT 10^6

Challenge Organism	Percent Mortality	Ave. Wt. grams	Ave. Length mm	% wt. of Controls	% Length of controls
Aeromonas hydrophila	**18**	**12.00**	104.2	**03.3**	**94.6**
Listeria sp.	12	15.50	110.65	107.6	100.5
Serratia **marcescens**	**10**	**12.70**	**108.2**	**88.2**	**98.2**
Pseudomonas fluorescens	4	14.87	106.2	103.3	96.4
Pasteurella multocida	4	13.62	109.0	94.6	99.0
Controls	2	14.40	110.15		

Figure 16

MORTALITIES EXPERIENCED BY EGG LOTS FROM FERTILIZATION THROUGH SAC FRY STAGE GROUPED ACCORDING TO THE PRESENCE OR ABSENCE OF *LISTERIA* SP. OR *AEROMONAS HYDROPHILA* WITHIN THE YOLK OF EGGS SAMPLED AT SPAWNING

	Green egg stage	Eyed Egg stage	Sac Fry stage	Cumulative mortality
Average mortality all egg lots	10.06%	23.46%	1.02%	31.87%
Average mortality all egg lots lacking *Aeromonas hydrophila* and *Listeria* sp.	4.73%	17.32%	0.82%	21.78%
Average mortality all egg lots with *Aeromonas hydrophila*	4.16%	28.86%	1.52%	32.97%
Average mortality all egg lots with *Listeria* sp.	10.94%	23.45%	1.08%	34.87%
Average mortality all egg lots containing both *Aeromonas hydrophila* and *Listeria* sp.	9.50%	52.54%	1.13%	56.13%